Journal of Medical Virology 56:112–117 (1998)

Interleukin 12 Enhances Deficient HCV-Antigen-Induced Th1-Type Immune Response of Peripheral Blood Mononuclear Cells

Jörg F. Schlaak,* Tanja Pitz, Hanns F. Löhr, Karl-Hermann Meyer zum Büschenfelde, and Guido Gerken

First Department of Medicine, University of Mainz, Germany

The aim of this study was to examine the possible immunomodulating effects of rhIL-12 on the immune response induced by different hepatitis C virus (HCV) antigens. Freshly isolated peripheral blood mononuclear cells (PBMC) of 33 patients with chronic HCV infection were stimulated with optimal concentrations of antigens from the NS3, NS4, NS5, and core region of HCV in the absence or presence of interleukin-12 (IL-12). Stimulation by α-CD3 + α-CD28, lipopolysaccharide (LPS), and pokeweed mitogen (PWM) were used as controls. Proliferation and cytokine production were determined by ^{3}H-thymidine uptake and enzyme-linked immunosorbent assay (ELISA) after 72 hr. After stimulation with antigen or antigen + IL-12, increased proliferation and production of interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) were observed in 23 of the 33 patients. Thus, a separation of the patients into HCV-antigen/IL-12 responders (group 1, n = 23) and HCV-antigen/IL-12 nonresponders (group 2, n = 10) was possible. Lower baseline IL-12- and LPS-induced IFNγ, TNFα, and IL-12 production was observed in group 2 due to a possible dysfunction of accessory cells. Significant antigen-induced Th2-type cytokine (IL-4, IL-10, IL-13) production was not found. According to clinical and serological parameters, group 2 comprised mostly patients with advanced liver disease. These data suggest an HCV-related cellular immune defect in patients with hepatitis C that can be restored in most patients by IL-12. ***J. Med. Virol. 56:112–117, 1998.***

KEY WORDS: cellular immune defect; proinflammatory cytokines; accessory cells

INTRODUCTION

The pathogenesis of the hepatitis C virus (HCV) infection and its individual clinical course are largely influenced by the host's immune response directed against the virus. Histologically, the infected liver is characterized by inflammatory infiltrations consisting of CD4$^+$ and CD$^+$ T lymphocytes and B lymphocytes often organized as focal lymphocytic aggregates [Bach et al., 1992; Scheuer et al., 1992; Freni et al., 1995]. While CD8$^+$ T lymphocytes are found predominantly in the vicinity of focal necrosis, CD4$^+$ T cells reside in portal and periportal areas [Liaw et al., 1995]. Liver-infiltrating CD8$^+$ T cells recognize various epitopes of the HCV in a major histocompatibility complex (MHC)-class I restricted manner with release of interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), GM-CSF, interleukin-8 (IL-8), and IL-10 [Koziel et al., 1992, 1993, 1995]. CD4$^+$ T cells from the peripheral blood and liver tissue with specificity for the core-antigen [Ferrari et al., 1994; Hoffmann et al., 1995] are correlated with disease activity and viraemia [Löhr et al., 1994b]. The recognition of the NS3 region by CD4$^+$ T lymphocytes of the peripheral blood is associated with viral elimination after acute hepatitis C [Diepolder et al., 1995]. Several researchers have shown that stimulation of peripheral blood mononuclear cells (PBMC) with HCV antigens induces Th1-type cytokine production [Löhr et al., 1994a]. Nevertheless, patients with chronic hepatitis C have a lower frequency of HCV-core antigen reactive IFNγ-producing T cells compared with anti-HCV positive healthy controls [Lechmann et al., 1996; Woitas et al., 1996].

The mechanisms leading to chronic hepatitis in most patients after infection with HCV remain to be determined. Because the clinical course is often characterized by an asymptomatic acute infection resulting in progressive liver disease over many years, it is postulated that an ineffective immune response cannot suppress viral replication or eliminate the virus. This in-

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant numbers: Schl 377/1–3, Lo 621/1–1, and SFB 311 A13.

*Correspondence to: Dr. Jörg Schlaak, First Dept. of Medicine, University of Mainz, Langenbeckstr. 1, 55101 Mainz, Germany.

Accepted 28 April 1998

sufficient immune reaction may be due to anergy or an ineffective cytokine profile of the antigen-specific T cells. Because IL-12 is known to induce Th1-type [Manetti et al., 1993; Schmitt et al., 1994], counteract Th2-type immune reactions [Kiniwa et al., 1992], and stimulate anergized T cells [Becker and Brocker, 1994], the aim of this study was to determine the effects of this cytokine on the in vitro recognition of various HCV antigens by mononuclear cells of the peripheral blood from patients infected with HCV.

MATERIALS AND METHODS

Patients

Freshly isolated PBMC of 33 patients with chronic HCV infection and 20 anti-HCV/HCV-RNA negative healthy controls were studied. The diagnosis was established by detection of anti-HCV antibodies [Alter, 1992] and HCV-RNA in the serum. The patients did not receive any immunomodulating drugs and had no history of prior IFNα therapy or current hepatitis B coinfection. All patients gave informed consent according to the 1975 Helsinki ethical declaration.

Antigens and Reagents

RhIL-12 was a kind gift of Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). α-CD3 and α-CD28 antibodies were purchased from Dianova (Hamburg, Germany). Lipopolysaccharide (LPS) was obtained from Sigma (St. Louis, MO). Recombinant HCV antigens from the NS3, NS4, NS5, and core region [Diepolder et al., 1995; Hoffmann et al., 1995] were purchased from Microgen (Munich, Germany). Tetanus toxoid was a kind gift from Behringwerke (Marburg, Germany).

HCV Genotyping and Serotyping

HCV serotyping was carried out using an enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (Murex Diagnostics Ltd., Temple Hill, England). HCV genotyping and subtyping (HCV 1-5, according to the nomenclature of Simmonds et al., [1993]) was undertaken using a line probe reverse hybridization assay (INNO-LIPA HCV, Innogenetics, Belgium).

Markers of HBV Infection

HBsAg, HBeAg, α-HBs, α-HBe, and α-HBc in serum were determined by commercially available ELISAs (Abbott Laboratories, Chicago, IL).

RNA Extraction

For isolation of viral RNA from frozen serum, 100-μl aliquots were digested for 10 min at room temperature in 800 μl of prewarmed lysis buffer [50 mM Tris-Cl (pH 8.5), 1 mM EDTA, 100 mM NaCl, 500 μg proteinase K, 0.5% SDS and 20 μg glycogen]. Then, RNA was extracted with phenol/chloroform and precipitated overnight with 1 ml 99% ethanol and 50 μl of 3 M sodium acetate (pH 4.7), followed by centrifugation. After two washing steps in 70% ethanol, the resulting RNA pellets were resuspended in RNAse-free water.

Quantitation of HCV RNA

The amount of HCV RNA present in 100 μl serum was determined using the Amplicor HCV monitor assay (Roche Molecular Systems, Basel, Switzerland) as described previously [Gerken et al., 1997]. The procedure was carried out according to the instructions of the manufacturer. The diluted RNA was added to equal volumes of master mixes (provided by the manufacturer). After a 1 hr 45 min incubation step in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer, Norwalk, CT), inactivation of potential carry-over amplicions, first-strand cDNA synthesis, and polymerase chain reaction (PCR) amplification were carried out in a single tube. Afterwards, the biotinylated and dUTP-containing PCR products were denaturated, hybridized to immobilized HCV probes, and detected colorimetrically using 96-well microtiter plates.

Isolation and Stimulation of PBMC

PBMC were isolated as described previously [Schlaak et al., 1993]. Briefly, PBMC were separated from heparinized blood by centrifugation on a Ficoll-Hypaque density gradient (Ficoll-Hypaque, Pharmacia, Uppsala, Sweden) at 350 × *g* for 20 min. Cells were collected and washed twice with phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum (FCS). The cells were then seeded into 96-well flat-bottom well plates (Greiner, Nürtingen, Germany) at a concentration of 2×10^5 cells/well and cultured in medium (RPMI, Biochrom, Berlin, Germany) containing 2% pooled human AB serum, 2 mM L-glutamine, and 50 μg/ml gentamicin in a humidified atmosphere at 37°C with 5% CO_2. The cells were cultured for 72 hr in the presence of optimal concentrations of the following stimuli: NS3 (2.5 μg/ml), NS4 (2.5 μg/ml), NS5 (2.5 μg/ml), core (1 μg/ml), tetanus toxoid (40 μg/ml), α-CD3 + αCD28 (1 μg/ml each), LPS (10 μg/ml), and pokeweed mitogen (PWM; 2 μg/ml). A medium control without stimulus was included in every experiment. In addition, antigen-induced stimulation was undertaken in the absence and presence of rhIL-12 (20 pg/ml). A concentration of 20 pg/ml IL-12 was chosen because higher amounts of IL-12 (tested up to 10 ng/ml) led to high background cytokine production and proliferation so that no synergistic effects with HCV-antigen stimulation could be observed.

Determination of Proliferation and Cytokine Production

After 72 hr of stimulation, supernatants were removed partially and stored at −20°C. Then, the cells were incubated with ^3H thymidine (0.25 μCi/well). After 24 hr, the incorporated radioactivity was measured by liquid scintillation counting to determine proliferative responses. The supernatants were tested for the presence of IL-4, IL-10, IL-13, IFNγ, and TNFα by ELISA as previously described [Koff and Dienstag, 1995]. All experiments were carried out in triplicate.

Statistical Methods

Statistical analysis was undertaken using the chi-square or 2-sample Wilcoxon test, respectively. Data are expressed as median ± SEM.

RESULTS

Influence of IL-12 on Spontaneous and HCV-Antigen-Induced Immune Responses of PBMC

The proliferative response and cytokine production (IL-4, IL-10, IL-13, IFNγ, and TNFα) of freshly isolated PBMC were studied in 33 patients with chronic hepatitis C after 72 hr of stimulation with recombinant antigens from the NS3, NS4, NS5, and core region of the HCV in the absence as well as in the presence of rhIL-12 (Fig. 3).

At a concentration of 20 pg/ml, IL-12 enhanced HCV-antigen-specific proliferation and production of IFNγ or TNFα at least 2-fold in 11 of 33 patients (33.3%). In 6 (18.2%) patients, only the combination of HCV antigens and IL-12 but not HCV antigens alone induced an immune response. In 6 other (18.2%) patients, HCV antigens induced cellular immune responses by PBMC to a level that could not be further enhanced by IL-12 (Fig. 1). In 10 (30.3%) patients, neither HCV antigen reactivity nor IL-12-induced up-regulation of the HCV-associated immune response were observed (Fig. 2).

On the basis of these data, the patients were divided into two groups. Group 1 represented HCV antigen responders (with or without IL-12 costimulation), whereas group 2 consisted of HCV antigen nonresponders (even in the presence of IL-12 costimulation). HCV antigen specific proliferation and cytokine induction were related mostly to stimulation with the core antigen. Because NS3, NS4, and NS5 induced only low level immune responses, if any, in our patients, these data are not shown. In none of the patients was HCV antigen-related Th2-type cytokine production (IL-4, IL-13) observed.

As described previously [Diepolder et al., 1995; Hoffmann et al., 1995], neither increased proliferation nor higher cytokine production were observed after stimulation of PBMC from anti-HCV antibody/HCV-RNA negative healthy individuals with HCV antigens in the presence and the absence of 20 pg/ml IL-12 (data not shown).

Freshly isolated PBMC of the HCV patients were stimulated with α-CD3 plus α-CD-28 antibodies and LPS, respectively, to study their proliferative and cytokine producing capacities. As shown in Table I, PBMC from group 2 (HCV-Ag NR) had significantly lower levels of LPS-induced IFNγ, TNFα, and IL-12 production and lower LPS-induced proliferative responses compared with group 1 (HCV-Ag R). Baseline IL-12 production was also lower in HCV Ag NR patients. In contrast, no differences were seen after T-cell stimulation with α-CD3 + α-CD28. Although statistically not significant, even higher median levels of IFNγ and TNFα could be observed in group 2.

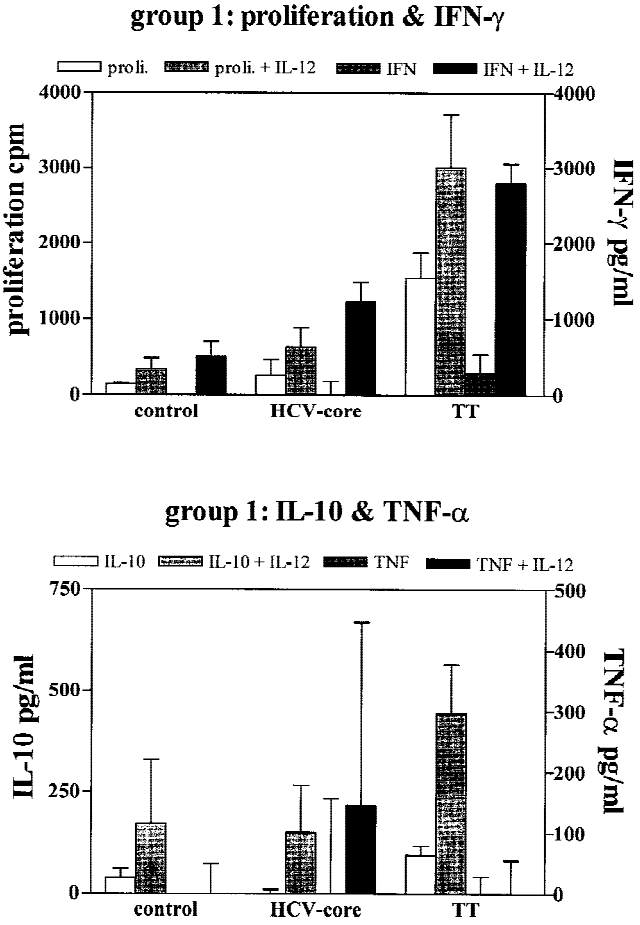

Fig. 1. IL-12 enhances hepatitis C virus (HCV)-antigen-induced proliferation, IFNγ, or TNFα, but not IL-10 production, in a subset of HCV patients. Freshly isolated peripheral blood mononuclear cells (2×10^5) were stimulated with optimal concentrations of HCV-core antigen and Tetanus toxoid (TT) in the absence or presence of 20 pg/ml rh IL-12. After 72 hr, proliferation and cytokine production were determined as described. Data are shown as median values ± SEM. All experiments were performed in triplicate. Note that the pooled data cannot reflect the substantial effects of IL-12 seen in individual patients (see Fig. 3).

Furthermore, the ability of IL-12 to modulate proliferation and cytokine production of the recall antigen tetanus toxoid (TT) was tested. These data show that IL-12 is able to enhance TT-induced proliferation, TNFα, or IFNγ production in both groups. Nevertheless, TT alone ($P = .003$) and the combination of IL-12 and TT ($P = .04$) induced significantly lower levels of IFNγ in group 2 compared with group 1 (Figs. 1 and 2).

Analysis of Clinical and Virological Parameters

After completion of the in vitro experiments, both groups were compared with regard to clinical and virological parameters (Table II). Group 1 patients had lower levels of γ-globulins, IgA and IgG but higher levels of thrombocytes and cholinesterase than patients from group 2.

There were no statistical differences between both groups with reference to age, sex, alanine transami-

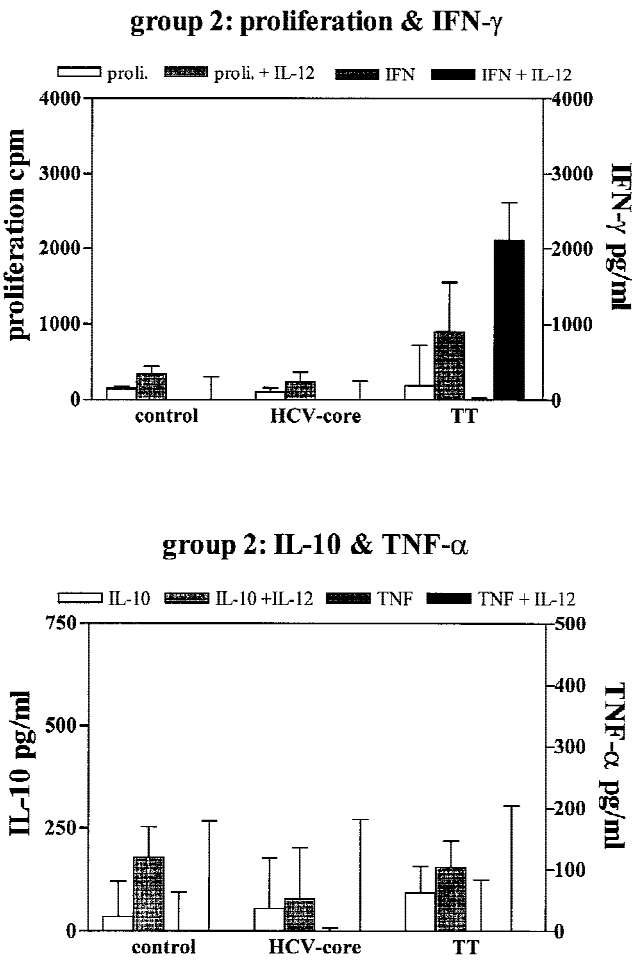

Fig. 2. IL-12 enhances Tetanus toxic (TT)-induced proliferation and IFNγ in peripheral blood mononuclear cells (PBMC) of patients who do not respond to HCV antigens. Freshly isolated PBMC (2×10^5) were stimulated with optimal concentrations of HCV-core antigen and TT in the absence or presence of 20 pg/ml rh IL-12. After 72 hr, proliferation and cytokine production were determined as described. Data are shown as median values ± SEM. All experiments were performed in triplicate.

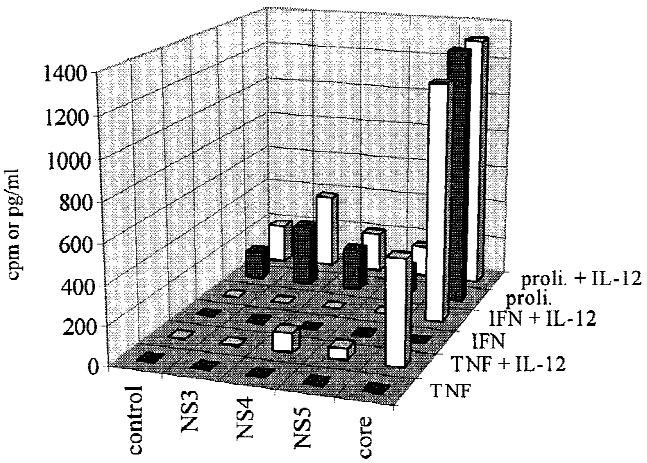

Fig. 3. An example for a patient from group 1 (HCV-Ag responder/IL-12 responder). Freshly isolated peripheral blood mononuclear cells (2×10^5) were stimulated with optimal concentrations of HCV antigens from the NS3, NS4, NS5, and core region in the absence and presence of 20 pg/ml rh IL-12. After 72 hr, IFNγ and TNFα production were determined as described. Data are shown as mean values.

nase (ALT), γ-GT, total bilirubin, IgM, previous hepatitis B infection, HCV copies in serum, duration of disease, and route of infection. The serological data suggested that group 2 mainly comprised patients with advanced liver disease. This diagnosis was confirmed when clinical and histological signs of cirrhosis were compared. Six of 10 patients (60%) from group 2 but only 3 of 23 patients (13%) from group 1 had histologically proven liver cirrhosis ($P = .003$). With regard to HCV geno- or serotype, there was a higher rate of geno- or serotype non-1 in group 1 (26%) compared with group 2 (10%), although this difference was not statistically significant.

DISCUSSION

Infection with HCV leads to chronic hepatitis in most patients and often culminates in the development of liver cirrhosis and hepatocellular carcinoma. The mechanisms underlying this process are still unclear. Because the clinical course is often only mild or even asymptomatic, it can be postulated that HCV antigen-specific immunocytes cannot mount a sufficient immune response against the virus.

We examined the HCV antigen-induced immune response in PBMC of patients with chronic hepatitis C. The data show that, in approximately two-thirds of the patients studied, HCV antigens alone or a combination of HCV antigens with IL-12 can induce proliferation and Th1-type, but not Th2-type cytokine production. Thus, patients with chronic hepatitis C can be divided into HCV Ag responders (group 1) and nonresponders (group 2). Group 2 patients probably represent those patients for whom an effective immune response against HCV cannot be mounted. It remains to be determined whether this unresponsiveness toward HCV antigens and IL-12 is the basis for the clinical finding that these patients do not profit from therapy with IFNα.

The state of HCV/IL-12 nonresponsiveness is not due to a general unresponsiveness toward IL-12, because IL-12 can enhance the immune response against the recall antigen tetanus toxoid in both groups. Group 2 patients, however, have significantly lower levels of TT-induced IFNγ production, whereas TT-induced IL-10 and TNFα production are not affected. With regard to immunological parameters, patients from group 2 have significantly lower levels of LPS-induced IFNγ, TNFα, and IL-12 production compared with group 1. Concerning clinical parameters, group 2 patients had more advanced liver disease. Nevertheless, this observation should not be overinterpreted due to the low number of patients in this group.

The mechanisms underlying the defective immune-mediated elimination of HCV are still difficult to define. An explanation for an insufficient immune response against HCV could be an "ineffective" cytokine

TABLE I. Comparison of Proliferation and Cytokine Production of PBMC From HCV-Ag/IL-12 Responders and HCV-Ag/IL-12 Nonresponders After Unspecific Stimulation

Parameter/stimulus	Group 1: HCV-Ag/IL-12 R (n = 23)	Group 2: HCV-Ag/IL-12 NR (n = 10)	P (1 vs. 2)
prolif./LPS	327 ± 69 cpm	176 ± 41 cpm	.02
IFNγ/LPS	428 ± 283 pg/ml	0 ± 137 pg/ml	.02
TNFα/LPS	408 ± 170 pg/ml	0 ± 134 pg/ml	.03
IL-12/control	419 ± 169 pg/ml	0 ± 83 pg/ml	.03
IL-12/LPS	694 ± 217 pg/ml	0 ± 25 pg/ml	.003

PBMC, peripheral blood mononuclear cells; HCV, hepatitis C virus; R, responder; NR, nonresponder; LPS, lipopolysaccharide; IFNγ, interferon-γ; TNFα, tumor necrosis factor-α.
Data are expressed as median values. There were no statistical differences between α- $CD3^+$ 28-induced cytokine production and proliferation and LPS-induced IL-4, IL-10, and IL-13 production. Statistical significance was calculated using the Wilcoxon test.

TABLE II. Analysis of Clinical and Virological Parameters

Parameter	Group 1: HCV-Ag/IL-12 R (n = 23)	Group 2: HCV-Ag/IL-12 NR (n = 10)	P (1 vs. 2)
γ-Globulins (%)	16.3 ± 1.4	29.8 ± 3.8	.002
Cholinesterase (kU/ml)	5.5 ± 0.42	2.8 ± 0.61	.002
Thrombocytes (x/nl)	228 ± 18	121 ± 25	.009
IgA (g/l)	2 ± 0.2	4.2 ± 1	.0003
IgG (g/l)	14.5 ± 1.3	23.8 ± 1.8	.01
Clinical signs of cirrhosis (histology, varices, pHT)*	yes: 3, no: 20	yes: 6, no: 4	.003

HCV, hepatitis C virus; R, responder; NR, nonresponder.
Data are expressed as mean values ± SEM or numbers, respectively. There were no statistical differences with regard to age, sex γ-GT, ferritin, total bilirubin, IgM, α-HBc, HCV geno- or serotype, HCV viremia, duration of disease, and route of infection. Statistical significance was calculated using the chi-squared* or Wilcoxon test.

profile by antigen-specific T cells. Some investigators assume that T cells reactive with HCV antigens display a Th2-like cytokine profile, although so far no published data are available that substantiate this theory. In our experiments, we have tested the supernatants from PBMC stimulated with HCV antigens from virtually all regions of HCV for the presence of the Th2 cytokines IL-4, IL-10, and IL-13 and could not detect any of these cytokines. Nevertheless, these cytokines could be found in high amounts after nonspecific stimulation.

In our opinion, the lack of a sufficient Th1-type immune response against HCV could be due to a lack of costimulatory IFNγ-inducing cytokines such as IL-12 (and possibly others), because in many patients HCV antigens can lead to substantial stimulation of the immune system in vitro only when this cytokine is added to the culture. This opinion is substantiated by the finding that PBMC from HCV-Ag R produce significantly higher baseline and LPS-induced IL-12. Interestingly, in all three HCV Ag NR patients for whom baseline IL-12 production was observed, LPS suppressed IL-12 production. Because differences with regard to cytokine production between both groups are only seen after stimulation of accessory cells (LPS) or antigenic stimuli, but not after selective stimulation of T cells (α-CD3 + α-CD28), the lack of PBMC to respond to HCV antigens could be due to the lack of accessory cells to produce sufficient amounts of costimulatory cytokines or to respond to these molecules.

In our system, IL-12 selectively enhanced antigen-induced Th1-type cytokine production but not the production of Th2 cytokines, although it increased background production of IL-10. Functionally, this enhanced Th1-type cytokine production may lead to clearance of the virus.

Remarkable similarities are observed when the data presented in this study are compared with results published previously on the role of IL-12 in the pathogenesis in HIV disease. Both infections have a high rate of chronicity, and the rate of "immunity" against the infecting virus appears to be low in both diseases, a finding that could be due to a suboptimal immune response against the respective virus. Interestingly, two studies have shown that IL-12 can restore the deficient immune response of PBMC against HIV peptides or recall antigens only in patients without advanced HIV disease [Landay et al., 1996; Uherova et al., 1996]. In our study, we observed the same phenomenon in as far as IL-12 could restore the immune response of PBMC against HCV antigens only in patients without end stage liver disease. Furthermore, PBMC infected with HIV produce significantly less Th1 type cytokines (IFNγ, IL-2) and IL-12 but not IL-1, IL-6, or TNFα when stimulated with *Toxoplasma gondii* antigens [Gazzinelli et al., 1995]. Thus, the immunosuppressive effects of both HIV and HCV appear to be selective for Th1 cytokines and might explain the high rate of chronicity of these infections.

In conclusion, we have shown that IL-12 can enhance

or restore the immune response against HCV-antigens of PBMC from a subset of patients with chronic hepatitis C. These data should therefore be taken into consideration for ongoing and starting phase I trials of IL-12 in the treatment of chronic hepatitis C. It would be of interest to correlate these in vitro data with clinical responses to IL-12 therapy and to test their predictive value for the efficacy of this therapy.

REFERENCES

Alter HJ (1992): New kit on the block: evaluation of second-generation assays for detection of antibody to the hepatitis C virus. Hepatology 15:350–353.

Bach N, Thung SN, Schaffner F (1992): The histological features of chronic hepatitic C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 15:572–577.

Becker JC, Brocker EB (1994): Prevention of anergy induction in cloned T cells by interleukin 12. Exp. Dermatol. 3:283–289.

Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, Eichenlaub D, Pape GR (1995): Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346: 1006–1007.

Ferrari C, Valli A, Galati L, Penna A, Scaccaglia P, Giuberti T, Schianchi C, Missale G, Marin MG, Fiaccadori F (1994): T-cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. Hepatology 19:286–295.

Freni MA, Artuso D, Gerken G, Spanti C, Marafioti T, Alessi N, Spadaro A, Ajello A, Ferrau O (1995): Focal lymphocytic aggregates in chronic hepatitis C: occurrence, immunohistochemical characterization, and relation to markers of autoimmunity. Hepatology 22:389–394.

Gazzinelli RT, Bala S, Stevens R, Baseler M, Wahl L, Kovacs J, Sher A (1995): HIV infection suppresses type 1 lymphokine and IL-12 responses to Toxoplasma gondii but fails to inhibit the synthesis of other parasite-induced monokines. Journal of Immunology 155: 1565–1574.

Gerken G, Knolle P, Jakobs S, Meyer zum Bueschenfelde KH (1997): Quantification and genotyping of serum HCV-RNA in patients with chronic hepatitis C undergoing interferon treatment. Archives of Virology 142:459–464.

Hoffmann RM, Diepolder HM, Zachoval R, Zwiebel FM, Jung MC, Scholz S, Nitschko H, Riethmueller G, Pape GR (1995): Mapping of immunodominant $CD4^+$ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. Hepatology 21:632–638.

Kiniwa M, Gately M, Gubler U, Chizzonite R, Fargeas C, Delespesse G (1992): Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. Journal of Clinical Investigation 90:262–266.

Koff RS, Dienstag JL (1995): Extrahepatic manifestations of hepatitis C and the association with alcoholic liver disease. Seminars in Liver Disease 15:101–109.

Koziel MJ, Dudley D, Afdhal N, Choo QL, Houghton M, Ralston R, Walker BD (1993): Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. Journal of Virology 67:7522–7532.

Koziel MJ, Dudley D, Afdhal N, Grakoui A, Rice CM, Choo QL, Houghton M, Walker BD (1995): HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. Journal of Clinical Investigation 96:2311–2321.

Koziel MJ, Dudley D, Wong JT, Dienstag J, Houghton M, Ralston R, Walker BD (1992): Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. Journal of Immunology 149:3339–3344.

Landay AL, Clerici M, Hashemi F, Kessler H, Berzofsky JA, Shearer GM (1996): In vitro restoration of T cell immune function in human immunodeficiency virus-positive persons: effects of interleukin (IL)-12 and anti-IL-10. Journal of Infectious Diseases 173: 1085–1091.

Lechmann M, Langhans B, Bär S, Woitas R, Leifeld L, Ihlenfeldt HG, Jung G, Effenberger W, Sauerbruch T, Spengler U (1996). Deficient secretion of Th1 cytokines in patients with chronic hepatitis C. Hepatology 24(4, Pt. 2):266A.

Liaw YF, Lee CS, Tsai SL, Liaw BW, Chen TC, Sheen IS, Chu CM (1995): T-cell-mediated autologous hepatocytotoxicity in patients with chronic hepatitis C virus infection. Hepatology 22:1368–1373.

Löhr HF, Schlaak JF, Gerken G, Fleischer B, Dienes HP, Meyer zum Bueschenfelde KH (1994a): Phenotypical analysis and cytokine release of liver-infiltrating and peripheral blood T lymphocytes from patients with chronic hepatitis of different etiology. Liver 14:161–166.

Löhr HF, Schlaak JF, Kollmannsperger S, Dienes HP, Meyer zum Bueschenfelde KH, Gerken G (1994b): Liver-infiltrating and circulating $CD4^+$ T cells in chronic hepatitis C: immunodominant epitopes, HLA-restriction and functional significance. Liver 16: 174–182.

Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S (1993): Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. Journal of Experimental Medicine 177:1199–1204.

Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM (1992): The pathology of hepatitis C. Hepatology 15:567–571.

Schlaak JF, Loehr H, Gallati H, Meyer zum Bueschenfelde KH, Fleischer B (1993): Analysis of the in vitro cytokine production by liver-infiltrating T cells of patients with autoimmune hepatitis. Clinical and Experimental Immunology 94:168–173.

Schmitt E, Hoehn P, Huels C, Goedert S, Palm N, Ruede E, Germann T (1994): T helper type 1 development of naive $CD4^+$ T cells requires the coordinate action of interleukin-12 and interferon-gamma and is inhibited by transforming growth factor-beta. European Journal of Immunology 24:793–798.

Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993): Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. Journal of General Virology 74:2391–2399.

Uherova P, Connick E, MaWhinney S, Schlichtemeier R, Schooley RT, Kuritzkes DR (1996): In vitro effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus type 1. Journal of Infectious Diseases 174:483–489.

Woitas RP, Lechmann M, Langhans B, Bär S, Ihlenfeldt HG, Jung G, Sauerbruch T, Spengler U (1996) Cytokine response to hepatitis C virus core peptides in patients with chronic hepatitis C infection and healthy anti-HCV seropositives. Hepatology 24(4, Pt. 2):266A.